



# Identification by PCR signature-tagged mutagenesis of attenuated *Salmonella* Pullorum mutants and corresponding genes in a chicken embryo model

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## ABSTRACT

A key feature of the fowl-specific pathogen *Salmonella* Pullorum is its vertical transmission to progeny via the egg. In this study, PCR signature-tagged mutagenesis identified nine genes of a strain of *S. Pullorum* that contributed to survival in the chicken embryo during incubation. The genes were involved in invasion, cell division, metabolism and bacterial defence. The competition index *in vivo* and *in vitro* together with a virulence evaluation for chicken embryos of all nine mutant strains confirmed their attenuation.

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## 1. Introduction

The *Salmonella* genus contains two species, *Salmonella enterica* and *Salmonella bongori*, and comprises 2610 serovars that are common vertebrate pathogens causing disease ranging from enterocolitis to systemic infections [1]. Although most serovars have a wide host range, a small number are adapted to specific hosts, such as *Salmonella enterica* serovar Typhi in humans, *S. Dublin* in cattle and *S. Gallinarum* and *S. Pullorum* in poultry.

As an avian pathogen, *Salmonella* Pullorum continues to produce severe systemic diseases of domestic poultry with economic losses worldwide arising from high morbidity and mortality and reduction in egg production [2–4]. *S. Pullorum* is thus a major factor restricting the growth of the poultry industry, especially in developing countries [5]. In addition to horizontal transmission in very young birds, *S. Pullorum* in infected hens may be transmitted vertically via the ovary to the egg, with subsequent extensive horizontal transmission in the hatchery. Little is known about bacterial factors that allow *Salmonella* Pullorum to survive in the chicken embryo during incubation without causing death of the embryo.

We employed PCR signature-tagged mutagenesis in use in our laboratory [6] to identify the genes associated with the survival of *S. Pullorum* in the chicken embryo.

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## 2. Materials and methods

### 2.1. Bacterial strains and chicken embryo

*S. Pullorum* SP S06004 is virulent for newly hatched chickens, and both this and a set of 12 donor *E. coli*  $\chi$ 7213 strains containing the tagged-plasmid pUTminiTn5 were grown in Luria-Bertani (LB) broth (Difco, USA) [6]. When required, this medium was supplemented with 1.5% (w/v) agarose, ampicillin (Ap, 100 mg/ml), kanamycin (Km, 50 mg/ml), and chloramphenicol (Cm, 34  $\mu$ g/ml).

### 2.2. Construction of the transposon mutant library

Following a previous protocol [6], each tagged-transposon with ampicillin, kanamycin and chloramphenicol resistant genes in the *E. coli*  $\chi$ 7213-pir strain, which requires 2,6-diaminopimelic acid (DAP) for growth, was transferred into the recipient S06004 by conjugation, with each donor containing the specific tag. The transformants of 12 mutant libraries were generated on selective LB agar plates containing 100  $\mu$ g/ml ampicillin, 50  $\mu$ g/ml kanamycin, and 34  $\mu$ g/ml chloramphenicol without DAP.

### 2.3. Identification of attenuated *S. Pullorum* mutants and corresponding genes

One mutant strain was selected randomly from each mutant library, and 12 mutant strains were pooled together to make the input

pool. From the input pool, 100 CFU/100  $\mu$ L and 200 CFU/100  $\mu$ L were injected separately into the allantoic cavity in 5 16-day-old embryos. Five days later the chickens were hatched, and all were killed humanely. The spleens and livers were removed aseptically and homogenized in deionized water. The homogenates were plated on selective media to form the output pool.

Chromosomal DNA was extracted from the input and output pools. PCR identification of the mutants was carried out, and PCR amplicons from the output pools were compared with those from the input pools. Any mutant not represented in the output pool was identified tentatively as an attenuated strain.

## 2.4. Virulence analysis of attenuated mutants

### 2.4.1. Growth phenotypes

Newly cultured salmonella mutants ( $1 \times 10^6$  CFU/ml) and the parent strain S06004 were cultured again in new LB media. Every 2 h, the bacterial concentration according to the OD600 was detected, and growth curves of the 9 mutants together with the parent strain were made and compared to each other.

### 2.4.2. In vivo competitive virulence assay

All of the attenuated mutants from the primary screen were confirmed by repeating the screening procedure using the individual strains. In addition, each mutant and parent strain were mixed together in equal numbers (100 CFU/100  $\mu$ L each) for an *in vivo* competition assay, in which the mixtures were administered to 5 16-day-old embryos via the allantoic cavity following a previously published protocol [6].

### 2.4.3. Virulence for 16-day-old embryos

Each attenuated mutant (100 CFU/100  $\mu$ L) was inoculated separately into the allantoic cavity of 16-day-old embryos ( $n = 10$ ). After 5 days, the degree of attenuation was measured by the hatch rate and absence of isolation of the mutant in the liver and spleen of the newly hatched chicken.

## 2.5. In vitro competitive index

For the *in vitro* assay,  $5 \times 10^3$  CFU of mutant and parent were co-inoculated in 5 ml LB. Cultures were grown at 37 °C for 12 h with shaking (180 rpm), and the input and output ratios of the mutant and parent strains were determined by selective plating as described above. The competitive index (CI) was calculated as the ratio of the mutant (CFU at hour 0/CFU at hour 12) divided by the ratio of the parent (CFU at hour 0/CFU at hour 12). For each mutant strain, the mean *in vitro* CI from two such experiments was recorded.

**Table 1**

Gene identification of mutants by flank sequences of the mini-Tn5 transposon.

Gene	Accession	Query coverage (%)	Functions	Competition index (CI)		
				<i>In vivo</i>	<i>In vitro</i>	
				Spleen	Liver	
<i>uvrY</i>	CP003047.1	100	invasion response-regulator	<0.001	<0.001	1.07
<i>folA</i>	CP003047.1	100	dihydrofolate reductase type I	0.11	0.56	0.72
<i>citC</i>	CP003047.1	100	[citrate (pro-3S)-lyase] ligase	0.11	0.58	0.45
<i>wza</i>	CP003416.1	99	polysaccharide export protein	<0.001	<0.001	0.57
<i>yabF</i>	CP003047.1	100	putative NAD(P)H oxidoreductase	0.74	0.68	5.52
<i>rfbG</i>	CP003047.1	100	CDP-glucose 4,6-dehydratase	<0.001	<0.001	2.52
<i>miaA</i>	CP003047.1	100	tRNA delta-2-isopentenylpyrophosphate (IPP) transferase	<0.001	<0.001	0.37
<i>ftsN</i>	CP003047.1	100	cell division protein	<0.001	<0.001	0.027
<i>unknown</i>	CP003047.1	100	alcohol dehydrogenase class III	0.23	0.3	0.31

## 3. Results

### 3.1. Establishment of the chicken embryo infection model of *S. Pullorum*

Sixteen-day-old chicken HY-line embryos were inoculated using an 18-gauge needle with 200 CFU/ $\mu$ L of input pools via the allantoic cavity, which was found to be optimal, resulting in 50% hatch rates.

### 3.2. Identification by PCR signature-tagged mutagenesis of attenuated *S. Pullorum* mutants and genes in the chicken embryo infection model

Twelve mini-Tn5 transposon mutant libraries were constructed; each library contained specific signal tag-labelled sequences encoding Km<sup>r</sup> and Cm<sup>r</sup> in the *S. Pullorum* genome as biomarkers. One tag-labelled mutant strain from each library was included in each input pool.

To model the effect of the mutants on embryos, 2 groups of 5 16-day-old chicken embryos were inoculated via the allantoic cavity with 100 CFU and 200 CFU of input pools, respectively. After the chickens were hatched, bacteria isolated from their livers and spleens were taken as output pools in which mutants were screened by PCR. A total of 100 input pools including 1200 mutants were screened. Nine mutants were identified as attenuated. From sequences flanking the transposon, transposon-disrupted genes were identified by a BLAST comparison with the bacterial genome in NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The genes identified in the 9 mutants shown in Table 1 are involved in invasion (*uvrY*::Tn5), cell division (*folA*::Tn5, *miaA*::Tn5, *ftsN*::Tn5), metabolism (*citC*::Tn5, *yabF*::Tn5, *rfbG*::Tn5, *unknown*::Tn5), and bacterial defence (*wza*::Tn5).

### 3.3. Growth phenotypes

Fig. 1a and b shows the growth of mutants in comparison with the parent strain in two experiments. The growth rates of all mutants were slower than that of S06004, further confirming the deleterious effects of the mutants on the *in vitro* metabolic characteristics.

### 3.4. Analysis of the virulence of individual mutants

The competitive index values obtained from the simultaneous inoculation of 16-day-old chicken embryos with each of the 9 mutants together with the parent strain are shown in Table 1. The virulence of these mutants for 16-day-old embryos is also shown.

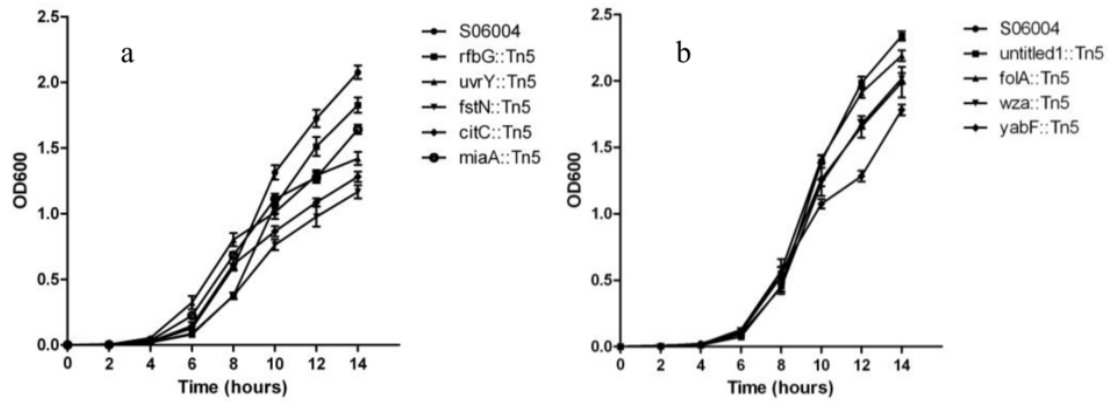


Fig. 1. Growth curves of the transposon mutants compared to S06004.

The *in vivo* competitive index data, calculated as the change in CFUs of the mutant 5 days after inoculation divided by the same change in the parent strain, showed that none of the mutants performed as well as the parent. Mutants *yabF*, *citC*, *folA* and the unknown::Tn5 all have CI values greater than 0.1 and in some cases much higher than that.

The virulence of *Salmonella Pullorum* mutants was assessed for 16-day-old chicken embryos ( $n = 10$ ) on the hatch rates and positive live chickens (Fig. 2). All 9 mutants showed different degrees of attenuation. The hatch rates for *rfbG*::Tn5, *yabF*::Tn5, *citC*::Tn5, *fisN*::Tn5 and *folA*::Tn5 were all greater than 50%. Mutant *rfbG*::Tn5 was not isolated from any of the hatched chickens, whereas the isolation rates for mutants *fisN*::Tn5 and *folA*::Tn5 were 87.5% (7/8) and 55.6% (5/9), respectively.

#### 4. Discussion

Among poultry-related *Salmonella* serovars, *S. Enteritidis*, *S. Pullorum* and *S. Gallinarum* are reported to show persistent infection with vertical transmission. *S. Pullorum* has been experimentally shown to persist in convalescent birds after infection in 2-4-day-old-chickens [7], and at the onset of the lay surge in sex hormones, it is believed to suppress T cell responsiveness [8], leading to its extensive spread to the reproductive tract, resulting in uninfected eggs, a large proportion of which will result in infected live chickens at hatch. The persistent infection is thought to result largely from a Th2-type

immune response directed by the pathogen in contrast to the characteristic Th1-type response induced by *S. Enteritidis* [9].

How applicable this model is to *S. Gallinarum* and *S. Enteritidis* is unclear. In some inbred chicken lines, *S. Gallinarum* shows persistent infection until 14–15 weeks of age, but experimental infection either results in death or no disease, depending on the genetic background of the birds [10]. The evidence for the vertical transmission of *S. Gallinarum* is largely epidemiological [2]. The immunological basis of infection with *S. Enteritidis* is largely unknown, although it has been shown experimentally that chicks that hatch from eggs derived from a *S. Enteritidis* infected flock show persistent infection until point-of-lay [11]. The genes required for the survival of *S. Enteritidis* within the egg have been identified previously [12].

The interaction between *S. Pullorum* and the developing embryo has not been investigated to any extent. The antimicrobial components in eggs, including lysozyme C, ovalbumin, ovotransferrin, ovomucin, zona pellucida (ZP) proteins, oviductin protease, two ATPases, and beta-defensin-11, have been known for some time [13]. In addition to the immunological relationship between the pathogen and host, the bacterial factors that lead to persistent embryo infection are completely unknown.

In this study, we used chicken embryos to screen a signature-tagged mutagenesis library of 1200 mutants of *S. Pullorum* SP S06004. This was done using 16-day-old embryos. Inoculation of embryos that were between 9 and 16 days of age resulted in death of the embryo.

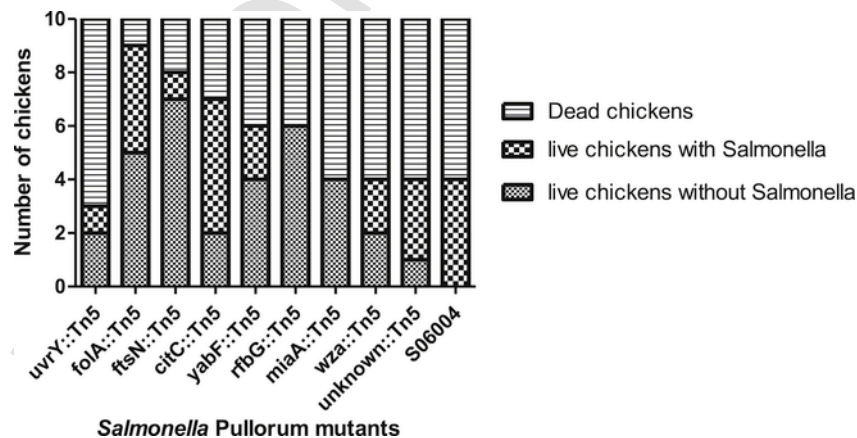


Fig. 2. Virulence assessment of *Salmonella Pullorum* mutants for 16-day-old chicken embryos ( $n = 10$ ) on the hatch and absence of isolation of each mutant in a newly hatched chicken.

Nine mutants were found to be attenuated by showing reduced survival in embryos. The genes affected were involved in invasion (uvrY::Tn5), cell division (ftsN::Tn5), metabolism (miaA::Tn5, yabF::Tn5, citC::Tn5, folA::Tn5, rfbG::Tn5, unknown::Tn5), and bacterial defence (wza::Tn5). The genes identified were different from those identified previously as being required for virulence in 2-day-old broiler chickens [6,14].

The uvrY gene is involved in the expression of type 1 fimbriae, which facilitates adhesion to various abiotic surfaces. uvrY has been shown to be beneficial to biofilm formation and the persistence and virulence of uropathogenic *E. coli* [15]. The uvrY gene encodes a DNA-binding response regulator of the BarA/UvrY two-component regulatory system, which responds to short chain carboxylates [16,17]. The hybrid sensor kinase BarA and its cognate response regulator UvrY, members of the two-component signal transduction family [18,19], activate transcription of CsrB and CsrC noncoding RNAs [20]. UvrY can regulate biofilm formation, motility and virulence determinants in uropathogenic *E. coli*. Deletion mutations of uvrY lowered expression of fimbrial recombinase genes, such as fimB, fimE, and fimA, and reduced transcription of virulence specific genes, such as papA, hlyB and galU. Swarming motility and expression of flhD and flhC were also diminished in the mutant [15].

The folA gene is involved in encoding dihydrofolate reductase (DHFR), which catalyses an electron-transfer reaction to form tetrahydrofolate, a carrier of single-carbon functional groups utilized in central metabolism, including de novo purine biosynthesis, dTTP formation, and methionine and glycine production [21]; folA-lacking bacteria will use flavin-dependent thymidylate synthases for deoxythymidine-5'-monophosphate synthesis [22].

The citC gene encodes a component of isocitrate dehydrogenase, which catalyses the reaction of oxidative decarboxylation from isocitrate to  $\alpha$ -ketoglutarate in the citric acid cycle, during which NAD<sup>+</sup> is reduced to NADH. Citrate utilization in *S. Typhimurium* is mediated by Na<sup>+</sup> dependent transporters (citC) belonging to the hydroxy carboxylate transporter family [23]. Deletion of the citC gene leads to a reduced capability of intracellular replication or survival compared to the wild-type strain of *S. flexneri* 2a in a HeLa cell infection model, demonstrating that the CitC protein could take part in bacterial virulence [24].

The yabF gene with the three genes orf5, sucB, fdhD encodes probable NAD(P)H oxidoreductases. YabF protein expressed by the yabF gene confers NAD(H)-dependent activity with short chain polyols or carbonyl compounds as substrates on *E. coli* clones [25]. Additionally, the YabF protein is required for the activity of the KefC glutathione-gated potassium efflux system in *Escherichia coli*; it is required for the maximum activity of KefC, which is a subunit of the KefC K<sup>+</sup> efflux system. The YabF protein can suppress the activity of KefC to control the outflow of K<sup>+</sup>. Deletion of the yabF gene reduces KefC activity up to 10-fold, and a supply of YabF in the complementary strain can restore bacterial activity [26].

The miaA gene encodes the MiaA protein, which is the consensus substrate required by the isopentenyl-tRNA transferase of *E. coli*. The enzymatic reaction involves a RNA-protein mutually induced fit mechanism in which large domain movements in MiaA provoke the partial unfolding of the substrate tRNA anticodon loop [27]. *Salmonella typhimurium* miaA mutants lacking the tRNA base modification cis-2-methylthioribosylzeatin (ms2io6A), in which leucine synthesis was defective, were examined and found to be sensitive to a variety of chemical oxidants and unable to grow aerobically at 42 °C in a defined medium [28].

rfbG, which is part of the rfb gene cluster of *Salmonella* LT2, encodes a CDP-glucose 4,6 dehydratase. It is essential for the expression of the O antigen [29]. rfb::Tn mutants of *Vibrio cholerae* O1, in-

cluding the rfbG::Tn mutant, were severely attenuated, as measured by both LD50 and their ability to compete with wild-type parents when analyzed in the infant mouse cholera model [30].

The ftsN gene encodes one of the 12 types of essential cell division proteins that are currently known. Recruitment of AmiC to the division ring is mediated by a N-terminal non-amidase targeting domain and requires the septal ring component FtsN [31]. FtsN is an essential cell division protein that interacts with the murein (Peptidoglycan) synthase PBP1B in *Escherichia coli* and stimulates the in vitro murein (Peptidoglycan) synthesis activities of PBP1B. Coordination of FtsN with PBP3, PBP1A, PBP1B and other cell division proteins enables completion of the cell division process, and the FtsN protein could play a role in controlling or modulating the activity of PBP1B.

In pathogenic *E. coli* E69, the Wza protein forms a pore in the outer membrane that transports the K30 capsular polysaccharide from its site of synthesis to the outside of the cell, which protects the bacteria in the host [32]. Homologs of Wza are widespread in Gram-negative bacterial pathogens in which capsules are critical virulence determinants. Wza spans the outer membrane using a barrel composed of amphipathic  $\alpha$ -helices, in contrast to a  $\beta$ -barrel, which is used by nearly all other outer membrane channels. The transmembrane helical barrel of Wza also forms the external opening to a hydrophilic translocation pathway that spans the periplasm [33].

Identification of the bacterial characteristics that are unique to *S. Pullorum* and allow the pathogen to survive within the developing embryo is not only important in understanding the immunomodulation that is associated with this pathogen but will also contribute to the development of more rational vaccines to protect the laying hen and the egg itself.

## Conflict of interest

The authors have no financial conflicts of interest.

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